Bio-degradable plastic production by bacteria isolated from marine environment and organic-waste

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May 10, 2013

CERTIFICATE

This is to certify that the project report titled **"Bio-degradable plastic production by bacteria isolated from marine environment and organic-waste"** submitted by **Mr. Pabitra Bhagowati** to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in LIFE SCIENCE is a bonafide record of work carried out by him under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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DECLARATION

I, Pabitra Bhagowati, hereby declare that this research project report entitled "***Bio-degradable plastic production by bacteria isolated from marine environment and organic-waste***" is the original work carried out by me under the supervision of Dr. Surajit Das, in the Laboratory of Environmental Microbiology and Ecology (LEnME), Department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge and belief the work reported here or any part thereof has not been presented to any other Institute or University for the award of any degree or diploma.

Pabitra Bhagowati
Date: 10-05-2013
Place: Rourkela, Odisha, India

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Pabitra Bhagowati

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LIST OF ABBREVIATIONS

g	Gram
µl	Microlitre
ml	Millilitre
l	Litre
h	Hour
°	Degree
C	Centigrade
min	Minute
LB	Luria Bertani
MHA	Muller Hinton Agar
%	Percentage
+	Positive
-	Negative
No.	Number
sp.	Species

ABSTRACT

Bioplastics are biomass based biodegradable plastics which can be derived from corn starch, pea starch, vegetable fats and oils as well as microorganisms like bacteria, algae etc. They may be used for packaging purposes and catering items like bowls, pots, straws, cutlery etc., for making bottles for soft drinks, bags, trays etc. Plastic is one of the major pollutants at present time around the world, which is used for daily use like packaging materials, carry bags, manufacturing of different types of materials etc. So, to replace the use of synthetic plastic as well as to reduce the increasing environmental pollution an alternative must be developed. This need of synthetic plastic can be fulfilled by use of bioplastics. Polyhydroxyalkanoates are polymers produced by bacteria among which Polyhydroxybutyrate (PHB) is one major group. The property of PHB is similar to synthetic plastics. So, it can be used as a suitable alternative to the present day conventional practices for sustainability. Several bacterial species like *Actinobacillus, Azotobacter, Agrobacterium, Rhodobacter* and *Sphaerotilius* have been under focus for their ability of converting organic waste to bacterial PHA. For industrial production of PHB, some bacterial species like *Bacillus* spp., *Pseudomonas* spp., *Aeromonas* spp., *Cupriavidus* spp. have been extensively used for their potential to produce PHB. Since the production of bio-plastic is expensive many techniques have been adopted for large scale production. But, to obtain PHB in large amount the selection of proper strains of bacteria, capable of producing or accumulating PHB is necessary. Marine ecosystem is one of the largest ecosystems on Earth and still required to be explored. So in this study, comparison of the production of PHB (Bio- Plastic) in Marine and Soil bacteria has been done to find out which one has the potency to accumulate more PHB.

Keywords: Bioplastic, PHB, Synthetic plastic, Bacteria, Marine.

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INTRODUCTION

1.1 Environment and Life

Life – a beautiful word that holds many information within itself. It can be said that it is a system or object with many characters like self-sustaining and signalling mechanisms which differentiates them from other objects. Those others are called non-living objects or non-living systems. A system, in biology, can be defined as a group of organs which associate together to perform certain task. In chemistry, a system can be defined as any object of universe which is under observation or study. The systems always remain associated with its surroundings or environment. This association of systems and also interaction among them and their environment forms an ecosystem. The environment has a major role in the development of a system as well in its existence. These phenomena are regulated by various biotic as well as abiotic factors of an ecosystem. For existence of a system in an environment, it should always maintain a balance with its surroundings by its activity. Occurrence of any disturbance in the balance between the biological systems and their environment leading to a hectic situation in which it becomes uncomfortable for the living systems to live. Nature always tries to maintain this balance whereas anthropogenic activities may disbalance the same.

1.2 Environmental Pollution and threat to life

Pollution is a condition in which contaminants are introduced in to the natural environments leading to adverse changes in the environment and human activity is the main cause for the same. Pollutants or contaminants are the components that cause pollution and thy may be foreign chemicals, substances (Fig. 1) or different forms of energy like heat, noise etc. Pollution may be point source or non-point source. The point source pollution is the type where pollution occurs in the same site where the pollutants are produced where as non-point source pollution is different from this type where the pollutants are carried to a different place from its origin via different transport media. Pollution may arise in different geographical locations leading to deformations in soil, water or air. Among different types, one newly discovered type of pollution is marine pollution, caused by various transport vehicles such as ship, ferry etc. and entry of various agricultural, industrial wastes into ocean water. Water from river and other water bodies flow and meet in the ocean. This carries various waste

materials which are harmful for the marine organisms and cause their death (Dash et al., 2013).

Pollutions may lead to critical problems in the global geochemical cycles as well as the sustainable habitation of humans as well as other organisms. Even though other organisms suffer from the adverse effects of natural changes, however, the main culprit is human. Various types of hazardous substances can enter the natural environment by a number of natural and/or anthropogenic activities, disturbing the living systems along with many adverse changes in the environment (Kampa and Castanas, 2008). In different urban areas huge megaplexes have been constructed which are not sustainable and they experience problems with waste management, heat islands, increasing pollution and crowding of increasing population etc. (William, 2011). CO_2 is toxic for pregnant women and when exposed, the fetus may be harmed. Likewise, car exhaust gases damage health of both adults and children, leading to change in behaviour and psycho-social development of children (Chelala, 2010; Markert et al., 2011).

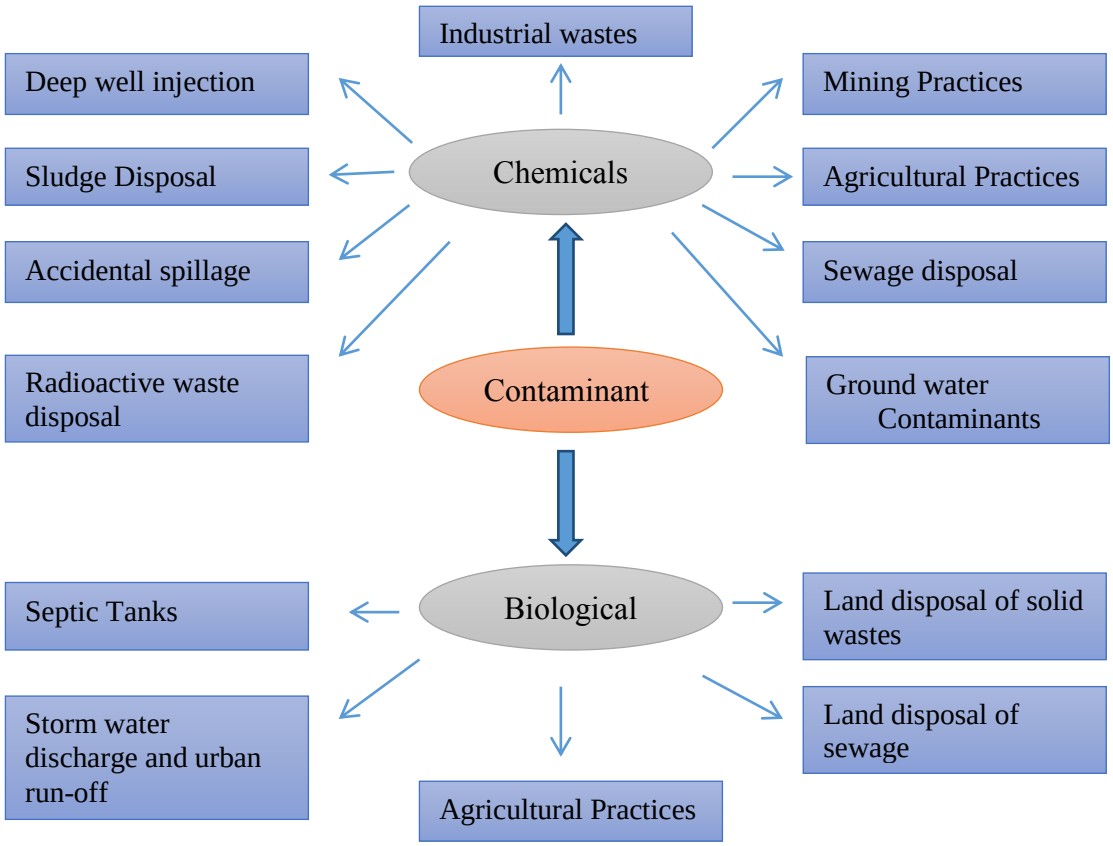


Fig.1.Various sources of contaminants

1.3 Environmental Pollution reported in India and World

Increased pollution over the surface of earth is creating critical problems in normal living conditions of human as well as other flora and fauna. The increase of temperature on Earth's surface is the result of ozone layer depletion and entrapment of greenhouse gases. In India, air quality data have been collected by NEERI (National Environmental Engineering Research Institute) from ten different cities of India such as Delhi, Kolkata, Mumbai, Chennai, Cochin, Kanpur, Nagpur, Hyderabad, Jaipur and Ahmedabad and from these data, Kolkata was found to be the most polluted city mostly with SO_2followed by Mumbai, Delhi, Ahmedabad, Kanpur, Hyderabad, Chennai, Nagpur and Jaipur. Jaipur was placed in the first position to be polluted with NO_x. SPM (Suspended Particulate Matter) level was found to be highest in Delhi and Kolkata and lowest in Mumbai and Chennai. In Delhi, air pollution level was found to be highest among all other cities. According to a report, level of SO_2 in atmosphere of Delhi has been recorded as 0.223ppm, whereas in Germany and USA 0.05 and 0.1 ppm are the permissible limits respectively. Methyl isocyanate leaked out from pesticide storage tanks in Bhopal, Madhya Pradesh, in 1984, killed over 3000 persons. The lead level of environment according to a guide of WHO is $2\mu g/m^3$ (Verma and Agarwal, 2004). Many cities of India and various countries of world have crossed this level of lead. Excess growth of phytoplankton was first observed in the water bodies of Europe and North America. Chemical wastes released from factories near Mirzapur, Uttar Pradesh has been reported to contain free chlorine which is the sole reason for the heavy mortality of fishes of Son River, Bihar.

In the big cities of India such as Mumbai, Delhi, Kolkata, Chennai, the contribution of vehicles to the air pollution is about 35%. A recent report on water pollution has described that daily around 29001million litres of liquid dirt are produced in India. In Punjab, India, during 2009, Uranium poisoning was detected, resulting from fly ash of thermal plants which led to birth defects in children of Bhatinda and Faridkot. To control noise pollution a new rule has been framed in the country that noise should not exceed the normal level of 65 decibel.

1.4 Plastic - a major environmental pollutant

Accumulation of non-degradable plastic bags in the environment is one of the major causes of pollution now- a- days. A statement, given by Supreme Court, says that plastic bags

threat is more serious than atom bomb. Only 1 to 2% of plastic bags in the USA end up getting recycled. Approximately 380 billion plastic bags are used in the United States every year that is more than 1,200 bags per US resident, per year. Approximately 100 billion of the 380 billion are plastic shopping bags. Thousands of marine animals and more than 1 million birds die each year as a result of plastic pollution. The United Nations Environment Programme estimates that there are 46,000 pieces of plastic litter floating in every square mile of ocean. Often mistakenly ingested by animals, clogging their intestines which results in death by starvation. Other animals or birds become entangled in plastic bags and drown or can't fly as a result and finally die. Plastics at present account for about 21% of all (paper, glass, tin plate. etc.) packaging materials. Packaging materials account for 25% of the total production of plastics in India, but in terms of consumption, they account for 52%. Plastic waste produced is around 2.0 million tonnes. Though plastics constitute only about 2.4 % (world average) of the total municipal solid waste, they are perceived as a major threat because of their long life and light weight. In India, plastic waste accounts for only 0.6% of municipal solid waste, whereas in urban areas of Kerala, it is as high as 4 – 6%. Plastic accounts for approximately 10% of solid waste (Heap, 2009) and contributes 80% of the wastes accumulating on ocean surface, land, shorelines etc. (Barnes et al., 2009).

1.5 Novel approach of production of Bio-degradable plastic

Bio-plastics are bio-based, biodegradable plastics with almost similar properties to synthetic plastics. Biodegradation can be explained as a chemical process during which micro-organisms that present in the environment convert materials into natural substances such as water, carbon dioxide, and compost. The term bio-based means the material is partly derived from biomass (plants). Synthetic plastics remain in the environment for long time as they are resistant to degradation (Aminabhavi et al., 1990). Bioplastics are made from variety of sources like polysaccharides, lipids and also proteins (Averous, 2004; Hernandez-Izquierdo and Krochta, 2008; Siracusa et al., 2008; Gonzalez-Gutierrez et al., 2009). A few examples of protein used as substrates for bioplastic production are soy protein (Mohanty et al., 2005; Tummala et al., 2006; Zheng et al., 2003; Gonzalez-Gutierrez et al., 2009), wheat gluten (Domenek et al., 2004; Gomez-Martinez et al., 2009; Jerez et al., 2005; Song and Zheng, 2008; Sun et al., 2008; Zuo et al., 2008; Gonzalez-Gutierrez et al., 2009), zein (Kim, 2008; Gonzalez-Gutierrez et al., 2009), rice and egg albumin (Jerez et al., 2007a,b; Gonzalez-Gutierrez et al., 2009). Platicizer, which is a rupturing agent added with proteins to increase

plasticity (Pommet et al., 2005; Gonzalez-Gutierrez et al., 2009). The petroleum based conventional plastics are non-renewable where the feed stocks are reinforced by carbon fibres (Williams et al., 2000). Renewable resource feed stocks of plastics include polymers derived from microbial culture reinforced with natural fibres such as cellulose, jute etc. (Bismarck et al., 2002). The accumulation of synthetic, petroleum derived plastics in the environment is a major cause of pollution. So the approach to produce plastic, which is an essential polymer used in our day to day life, using microbes (product of microorganisms) is a novel approach. It will reduce the environmental pollution as well as the use of petroleum to make plastic bags. So it can be said in one word that bio-plastic is eco-friendly.

1.6 The present status of Bio-plastics and its future

Since the large scale production of Bio-plastic in industry is very much costly so it has not been used extensively. During 20^{th} century the bioplastics production was mainly dominated by the developed countries like North America, Japan, Western Europe etc. On the basis of this study, it has been assumed that, by 2013, Brazil will become one of the world's leading bioplastics producers. In Japan, the demand of bioplastics will reach a value six times more than 178000 metric tons in 2013. China has planned to produce 100000 metric tons of bioplastics by 2013. The market of bioplastics is in the nascent stage in Southeast Asia. A research work carried out by BCC has revealed a fact that the bioplastics market value has reached 541 million pounds in 2007. By 2012, this value is expected to reach a level of 1.2 billion pounds. In 2008, a number of Biodegradable plastics like polylactic acid, resins, polyesters etc. accounted for about 90% of total bioplastics demand. Biodegradable plastics are environment friendly and can replace all plastics products available at this time. Production of bioplastics will definitely result in reduction in emission of CO_2 compared to traditional plastics. A fear of damaging already existing recycling projects by the bioplastics is one of the major concerns. The cost of production of bioplastics is also too high. This is one of the major problems related to bioplastics development. The cost is around 1.3 to 4 Euro per Kg now.

REVIEW OF LITERATURE

2.1 Bioplastic: its synthesis and degradation by microbes

Bioplastics are biomass based biodegradable plastics which can be derived from corn starch, pea starch, vegetable fats and oils and microorganisms like bacteria, algae etc. They are used for packaging purposes and catering items like bowls, pots, straws, cutlery etc., for making bottles for soft drinks, bags, trays etc. (Fig. 2).

Fig.2. Biodegradable utensils, bottles, packaging materials made from bioplastics

It includes different types of plastics such as cellulose based, starch based, some aliphatic polyesters like Polylactic acid (PLA), Poly hydroxyl butyrate (PHB) etc. Poly-3- hydroxy butyrate (PHB) is 100% biodegradable and it is produced from various renewable sources (Godbole et al., 2003). It has similar physical properties with polypropylene. Due to this character, PHB is being able to attract the vision of researchers towards its study and production. Another reason for gaining priority is that use of these biodegradable and bio-based plastics will definitely reduce the pollution caused by CO_2 emission from plastic wastes (Numata and Doi, 2012).Poly-3-hydroxybutyrate (PHB) is a polymer of 3-hydroxybutyrate and are intracellular granules produced by prokaryotic organisms as energy and carbon storage during starvation (Schubert et al.,1988)(Fig.3).

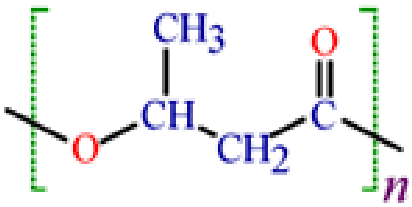


Fig.3. Chemical Structure of Poly-3-hydroxybutyrate (PHB)

Poly-3-hydroxybutyrate is included in the family 'Polyhydoxyalkanoates'. Accumulation of Poly-3-hydroxybutyrate in most of the microorganisms takes place in the presence of excess carbon and limited nitrogen sources (Verlinden et al., 2007; Singh and Parmer, 2011).

Biochemical studies have revealed two different pathways for synthesis of PHB.

(i) In organisms like *Azotobacter beijerinckii* and *Zoogloea ramigera*, a three-step metabolic pathway is seen. The first step is catalysed by enzyme 1-ketothiolase, which condenses acetyl coenzyme A (acetyl-CoA) to acetoacetyl-CoA. This intermediate is then reduced to D-(-)-P3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA reductase (Nishimura et al., 1978; Schubert et al., 1988). The last step is catalysed by the enzyme PHB synthase and cause head-to-tail polymerization of the monomer to PHB.

(ii) In *Rhodospirillum rubrum* PHB synthesis is carried out through five-step synthetic pathway. An NADH-dependent acetoacetyl-CoA reductase enzyme catalyses the formation of L-(+)-3-hydroxybutyryl-CoA, which is then converted to D-(-)-P-hydroxybutyryl-CoA by two stereospecific enoyl-CoA hydratases before polymerization (Moskowitz and Merrick, 1969; Schubert et al., 1988).

In contrast to β-ketothiolase and acetoacetyl CoA reductase, PHB synthase is the most important enzyme of the synthetic pathway. In *Bacillus megaterium* (Merrick et al., 1999), R. *rubrum* (Merrick et al., 1999), and *Z. ramigera* (Fukui et al., 1976; Fukui et al., 1982), it has been observed that PHB synthase is associated with phospholipids on the surface of the PHB granules under certain conditions of growth.

Especially in membrane fractions the activity of PHB synthase enzyme activity has been found and the activity increases in absence of nitrogen. PHB synthase activity is not affected by antibiotics like Chloramphenicol which inhibits protein synthesis. It has been found that acetyl phosphate addition into cell free extracts from cells not starved with nitrogen increases the activity of the enzyme. The Km value of PHB synthase enzyme is found less which helps in production of PHB since it becomes active in low concentration (Miyake et al., 1997).

In absence of nitrogen PHB synthesis generally increases. The reasons may be during nitrogen starved conditions reduce amino acid synthesis accompany increase in Acetyl CoA and the activity of Phosphoacetyltransferase (β- Ketothiolase) increases but the intermediate process and regulation mechanisms are yet to find out. In this starved condition concentration of acetyl phosphate increases and finally PHB synthase enzyme is activated (Asada et al., 1999).

Almost nothing is known to the scientists about the mechanism of the synthase enzyme reaction and other properties related to it. Griebel and Merrick (1971) proposed a protein, A-I, which in *Bacillus megaterium* mediates reaction between the monomer and the growing chain of the polymer and functions as an acyl carrier.

It has been observed from experiments that acetyl CoA acyltransferase is the main enzyme which regulates the synthesis of PHB.

The PHB synthesis begins with the condensation of two acetyl-CoA molecules to acetoacetyl-CoA by enzyme ketothiolase, encoded by the *phaA* gene (Fig. 5). This intermediate is then reduced to D-(-)-3-hydroxybutyryl-CoA by the enzyme named as acetoacetyl- CoA reductase, which is a product of the *phaB* gene. Finally, the enzyme PHA synthase encoded by *phaC* gene catalyses the polymerization of 3-hydroxybutyryl CoA to Polyhydroybutyrate by joining PHB monomers through the use of two thiolate groups (Steinbuchel et al., 2001).

Excess carbon source and exhaustion of any nutrient in the culture media like N_2, O_2, PO_4 increases production of PHB. Under normal growth conditions, acetyl CoA is used up in the TCA cycle, and resulting CoA inhibits the enzyme acetyl Co A acyl transferase as well as PHB synthesis. But during carbon excess and nutrient limitation NADH concentration increases by decreasing the activity of NADH oxidase. Increase NADH concentration decreases the activity of citrate synthase and isocitrate dehydrogenase and acetyl CoA level increases. Condensation of acetyl CoA to acetoacetyl CoA initiates PHB synthesis (Oeding and Schlegel, 1973; Jackson and Dawes, 1976; Page and Knosp, 1989) (Fig. 4). Increased NADH/NAD ratio is adjusted by PHB synthesis and PHB performs the role of electron acceptor (Oeding and Schlegel, 1973; Page and Knosp, 1989).

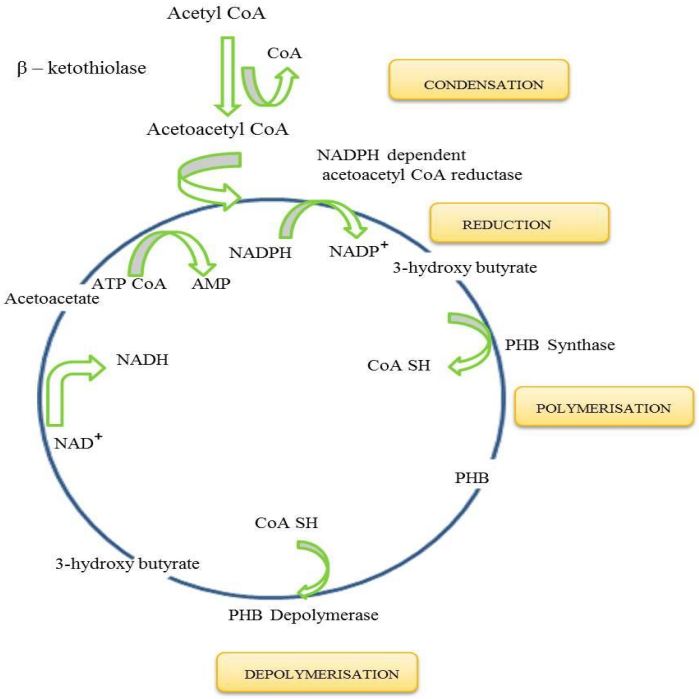


Fig.4. The cycle representing the synthesis and degradation of poly-hydroxy-butyrate (PHB)

The residues of PHA synthase have been identified and are found to be highly conserved across different microorganisms capable of producing PHA. The conserved residues are: Ser-260, Cys-319, Gly-322, Asp-351, Trp-425, Asp-480, Gly-507, and His-508. Residues like Cys-319 and Gly-322 are part of the motif G-x-C-x-G-G, required for the catalytic activity of PHA synthase enzyme. PHA synthase activity is diminished when in the highly conserved Cys-319 residue mutation occurs. It suggests that it is one of the thiolate groups.

The enzyme PHA synthase forms a dimer with the first thiol group (Cys-319) on one subunit which acts as the loading site according to the proposed model for PHA synthase function, and the same thiol group acts as the elongation site on the other subunit. The first thiol group binds to D-(-)-3-hydroxybutyryl-CoAcovalently and results in the liberation of coenzyme A.

In the same way, the corresponding thiol group present on the other subunit covalently binds to another molecule of D-(-)-3-hydroxybutyryl-CoA and performs the cleavage of coenzyme A on that particular molecule.

Then the subsequent D-(-)-3-hydroxybutyryl, attached to the second thiol group becomes the site for nucleophile attack. This activates D-(-)-3-hydroxybutyryl and joining of D-(-)-3-hydroxybutyryl on the first thiol group to the end of the monomer present at the

second thiol group by a trans-esterification reaction (Rehm, 2003). Finally, the process of elongation occurs to create high molecular weights polyesters (Steinbuchel et al., 2001)

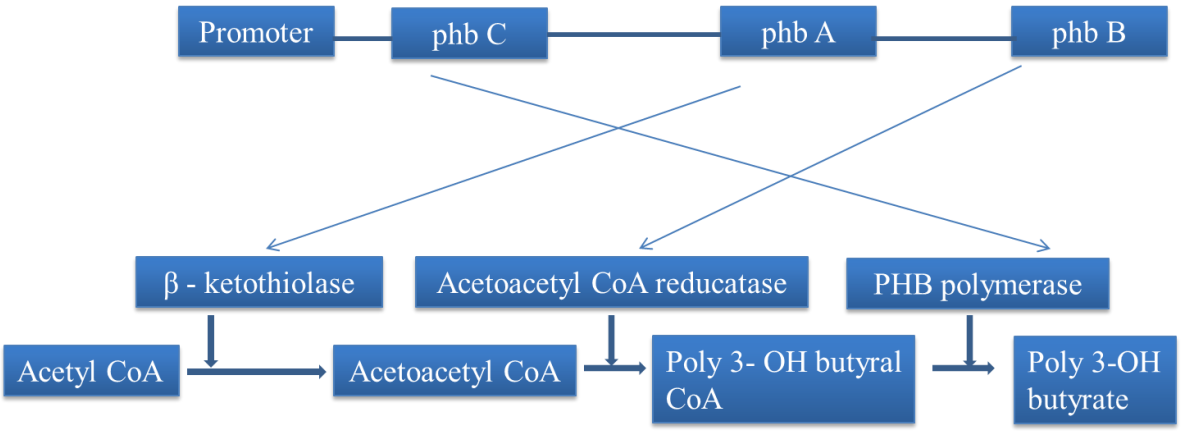


Fig.5. Operon model of genes responsible for production of PHB

During PHB accumulation, in the first step acetyl-CoA flux increases because of reduced amino acid synthesis derived from nitrogen starvation and phosphoacetyltransferase activity also increases. Increase concentration of acetyl phosphate, activates PHB synthase to synthesize PHB (Asada et al., 1999) (Fig. 6).

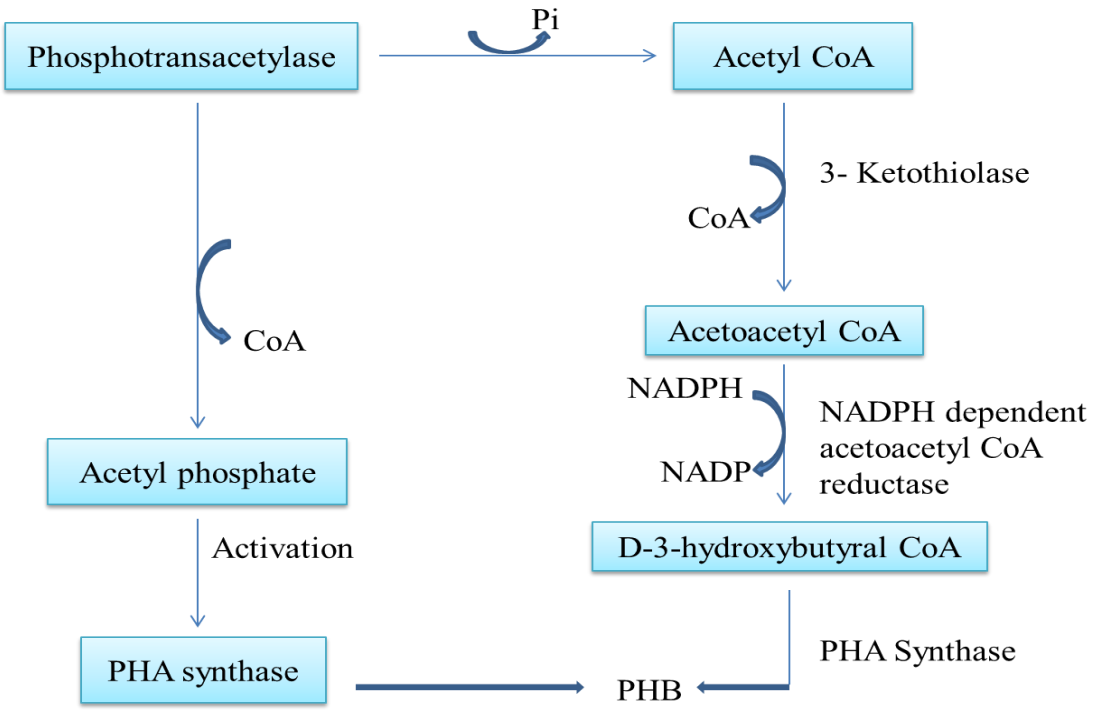


Fig.6.Hypothesized regulation in PHB metabolism

2.2 The history of bioplastic production

Poly-3-hydroxybutyrate was first detected by Lemoigne in 1926 from the Pasteur Institute, France (Lemoigne, 1926; Schubert et al., 1988). Poly3-hydroxybutyrate (PHB) is produced by joining of β- Hydroxybutyrate monomers by ester bonds. Since 1926, over 100 PHAs have been identified from different microbial species present in the environment (DiGregorio, 2009). Until 1980s, scientists were not able to find out any alternative for petroleum based plastics to reduce the pollution. In the late 80s, Anthony Sinskey from Massachusetts Institute of Technology (MIT) and his colleagues successfully isolated the first enzyme 'thiolase' which plays a major role in the biological process to produce bioplastics followed by the discovery of the genes required for the synthesis. The first patent applications of bioplastics were made in 1987 and finally accepted in1993 (DiGregorio, 2009).

2.3 Microbes as the bioplastic producers

Microbes have been reported to be the potent producers of PHB due to their high adaptability in various extreme environmental conditions. Out of these, *Bacillus* spp., *Pseudomonas* spp. and *Vibrio* spp. are found to be more efficient for PHB production due to their higher stability and reproducibility under environmental stress. Some of the major groups of potential bioplastic producers have been discussed below.

2.4 Efficient bioplastic producers

Many types of bacteria, such as *Bacillus* spp., *Pseudomonas* spp., *Cupriavidus* spp., and *Aeromonas* spp., have been studied for their use in industry for efficient capacity to produce PHA (Shimamura et al.,1994; Abe et al.,1994; Saito and Doi, 1994; Fuchtenbusch et al., 2000). Some bacterial species like *Bacillus megaterium*, *Ralstonia eutropha* have gained more attraction from the researchers. The PHB production from *Bacillus megaterium* has been reported to be around 84% (Prasanna et al., 2011).

Several bacterial species like *Actinobacillus, Azotobacter, Agrobacterium, Rhodobacter* and *Sphaerotilius* have been under focus for their ability of converting organic waste to bacterial PHA. For industrial production of PHB, some bacterial species like *Bacillus* spp., *Pseudomonas* spp., *Aeromonas* spp., *Cupriavidus* spp. have been extensively used for their potential to produce PHB (Shimamura et al., 1994; Abe et al., 1994; Saito and Doi, 1994; Fuchtenbusch et al., 2000; Numata and Doi, 2012).

2.5 Marine microbes as the potent bioplastic producing agents

Marine bacteria have recently attracted attention as potentially useful candidates for the production of PHAs. The advantages of using marine bacteria for the biosynthesis of poly hydroxyalkanoates (PHA)is because of avoiding contamination with bacteria that lack salt-water resistance, its ability to use filtered seawater as a culture medium, and the potential for production of extracellular PHA, and these all would contribute to large-scale industrial production of PHA(Numata and Doi, 2012).The main advantage of biodegradable polymers is that anaerobic microbes completely degraded to water, carbon dioxide and methane in various environments such as soil, sea, lake water and sewage and so it is disposable without harm to the environment (Brandl et al., 1988).

Although a few kinds of marine bacteria have been investigated for PHA production under some marine conditions, characterization have not been done in details of the resultant PHAs (Gonzalez-Garcia et al., 2008; Wang et al.,2010; Lopez et al.,2009; Numata and Doi,2012).

Some haloarchaeal species belonging to genera like *Haloferax*, *Haloarcula*, *Natrialba*, *Haloterrigena*, *Halococcus*, *Haloquadratum*, *Halorubrum*, *Natronobacterium*, *Natronococcus* and *Halobacterium* have found to be efficient producer of PHB (Poli et al., 2011).

Bacterial genera like *Beneckea* and *Vibrio* have been found to be first reported potent producers of PHA isolated from marine sediments (Lopez-Cortes et al., 2008).

2.6 The optimum conditions for bioplastic synthesis and influence on changes of parameters

PHB are lipid intracellular lipid granules which are formed by bacteria under stress conditions like limitations of nutrients such as nitrogen, phosphorus, oxygen etc. and in excess of carbon (Bitar and Underhill, 1990; Sindhu et al., 2011). Generally, in the production of PHB along with both presence and absence of nutrients other factors like initial culture pH, culture temperature, rate of agitation (*culture invitro or in industries*).

2.6.1 Effect of culture pH: Metabolic processes require specific pH to occur and slight change in pH affect the processes and make those critical (Wei et al., 2011). Wei et al. (2011) has also shown that the production of PHB is maximum at pH 7.0. The results obtained by Wei et al. (2011) are consistent with Palleroni and Palleroni,

(1978) where the pH range for maximum PHB production was recommended as 6.0-7.5.

2.6.2 Effect of culture temperature: Temperature also play a major role in PHB production. Wei et al.(2011) has shown that the PHB production is maximum at 30˚ C.

2.6.3 Effect of Agitation rate: Agitation rate also determines the growth of potent bacterial strains and PHB production. Proper agitation prevents the clumping of cells into large mass and thereby helps in the growth. Agitation facilitates each cell to utilize the nutrients available in the culture media. According to Wei et al. (2011), the rate of agitation should be in between 150-200 rpm and if it exceeds 200 the production decreases because of excessive shear force due to agitation.

2.7 Industrial production of bioplastics

Even though the large scale industrial production of bioplastics is costly, researcher are working to find out a better production by some potent PHB producing microorganisms using various types of substrates. According to Kumar et al. (2004), bacterial species present in activated sludge generated from a food processing industry are found to be potent for production of PHB. Bonartseva et al. (1994) has shown that maximum PHB accumulates in *Rhizobium lupine* when grown in presence of glutamate and mannitol. Feed batch culture is one of the popular methods to obtain high cell density and large amount of desired product (Wang and Lee, 1997). Wang and Lee (1997) have shown that nitrogen limited condition along with continuous feeding of sucrose increases the production of PHB.

2.8 Blending of substrates with PHB (bioplastics) to reduce the cost of production

The cost of industrial production of bioplastics is very high in comparison to synthetic plastics now-a-days and basically the cost of production depends on the cost of biomass for fermentation (Sindhu et al., 2011), but at the same time its production in large scale is also essential. So to reduce the cost to some extent blending of PHB can be performed with other polymers. According to Godbole et al. (2003), if the ratio of starch blending to PHB is maintained at 30:70 % it would be advantageous to reduce the cost of PHB.

OBJECTIVES

1. Isolation and screening of bacterial species capable of producing PHB from marine environment and organic wastes sources.

2. Characterization of PHB produced by the isolates.

3. Phenotypic and genotypic characterization of the PHB producing isolates.

4. To compare the PHB producing capability of the isolates.

5. To deduce the genetic mechanism of PHB production in the isolates.

MATERIALS AND METHODS

4.1 Isolation of bacterial isolates from sources like marine and organic-wastes

4.1.1 Isolation of marine bacteria: Ten different types of marine bacteria were isolated from study sites of Bay of Bengal along the Odisha cost. The two sites from where samples were collected include Chilka (19°44.582' N & 85°12.768'E) and Paradeep (20˚ 17.542'N & 86˚ 42.996'E) (Fig. 7). The samples were collected in falcon tubes and carried to the laboratory by keeping them on ice. The samples were then processed in the laboratory by serial dilution followed by spread plating in nutrient agar (Peptic digest of animal tissue 5g/l, Sodium Chloride 5g/l, Beef extract 1.5g/l, Yeast extract 1.5g/l, Agar 1.5%, pH-7.4±0.2) plates to get some isolated colonies. The spread plating was followed by incubation of the bacterial culture plates at 37˚ C for 24h. When the growth was proper, loop full cultures were taken from each single colony and streaked on culture plates containing nutrient agar medium to obtain pure culture of the isolates and this was followed by incubation of the plates at 37˚ C for 24h. The pure cultures were preserved and maintained by sub-culturing the isolates at an interval of 1-2 weeks.

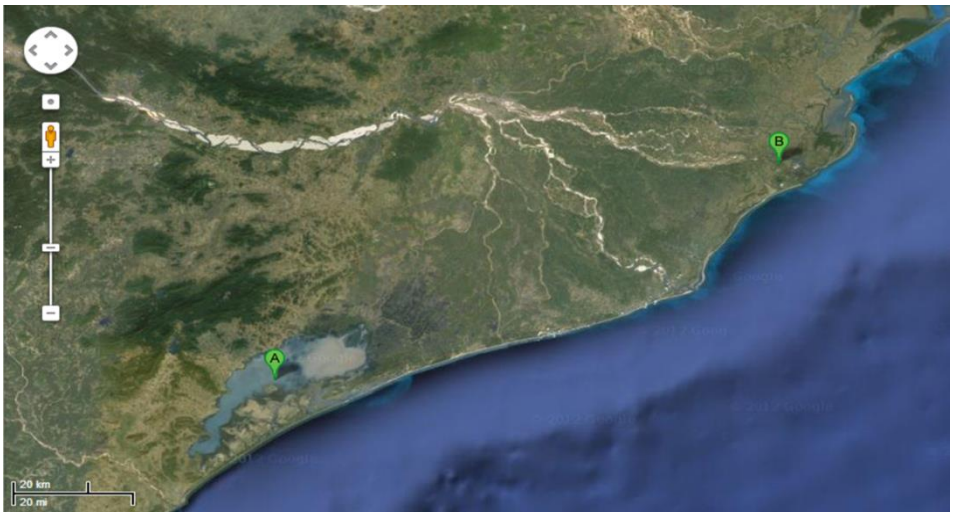


Fig.7. Collection sites of Marine water and sediment samples (A) Chilka, (B) Paradeep

4.1.2 Isolation of bacteria from Organic-Wastes: 20 different types of soil bacteria were isolated from organic-wastes samples collected from the two garbage dumping sites located at the North-West and South-East corners of National Institute of Technology, Rourkela campus (Fig. 8). After collection of samples, serial dilution was performed followed by spread plating of the diluted samples in nutrient agar plates and incubated the bacterial culture plates at 37° C for 24h. When the growth was observed in the plates, loop full cultures from different colonies were taken and streaked on culture plates containing nutrient agar medium as in the isolation of marine bacteria for obtaining pure culture of different isolates and incubated the plates at 37° C for 24h. The pure cultures of different isolates of organic-wastes bacteria were preserved (Fig. 10) for future use in screening for production of bioplastic and maintained by sub-culturing the isolates at an interval of 1-2 weeks same as the marine isolates.

Fig.8.Collection of samples from waste dumping sites located at South-East (A) and North-West (B) corner of NIT Campus

4.2 Screening of different isolates of marine and organic wastes bacteria for production of bioplastic

To screen the cultivated marine and organic wastes bacterial isolates Nile Blue staining was performed. Bacterial isolates were cultured for 2-3 days at 37° C in Minimal Davis Media (Dipotassium phosphate 7g/l, Monopotassium phosphate 2g/l, Sodium citrate 0.5g/l, Magnesium sulphate 0.1g/l, Ammonium sulphate 1g/l, pH-7.0±0.2) supplemented with dextrose (10ml of 10% in 1l of Minimal Davis Media) as carbon source. From each, a

loop full culture was taken on clean, sterile glass slides and heat fixed followed by staining with Nile blue stain. The samples were allowed to get stained for 20 min at room temperature and then slides were washed with sterile water. Then the slides containing the samples were allowed to air dry for few minutes and observed under fluorescence microscope at wavelength 490 nm. PHB granule producing bacterial isolates flourish bright yellowish-orange color (Ostle and Holt, 1982).

4.3 Extraction of produced PHB in the potent isolates

Two bacterial isolates, one from marine source (CS605) and another from organic wastes (SE1) were selected for further study of production of PHB based on intensity of brightness of the PHB granules produced by them. They were cultured in Minimal Davis Media supplemented with dextrose as carbon source for 3 days at 37° C at 150 rpm in a rotary shaker (Fig. 9). After 3 days of incubation, extraction of PHB was performed following sodium hypochlorite-chloroform method.5 ml of culture was centrifuged at 10,000 g for10 minutes and supernatant was discarded. The pellet was suspended in 2.5 ml of 4 % sodium hypochlorite for digestion and 2.5 ml of hot chloroform and was incubated at 37°C for 1 hour. The suspension was centrifuged at 1500 g for 10 minutes (The upper phase contains hypochlorite solution and the middle phase contains chloroform with cell debris).The bottom phase containing PHA with chloroform was collected and further was followed by extraction with hot chloroform and precipitated with ethanol and acetone (1:1).The precipitate was allowed to evaporate for dryness at 30° C to obtain PHA crystals (Singh and Parmar, 2011).

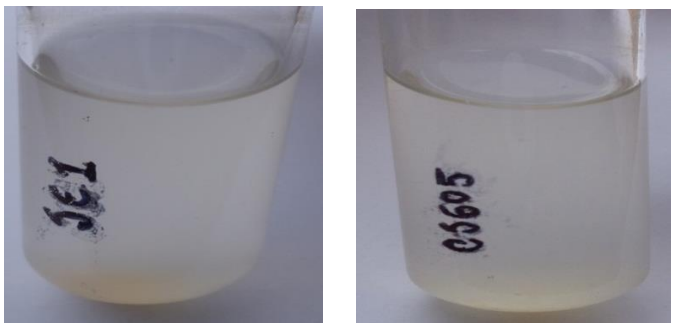


Fig.9.Culture of the two isolates in Minimal media for extraction of PHB

4.4 Characterization of extracted PHB by FTIR analysis

Extracted PHB samples were mixed with 2% KBr. Then the mixtures were compressed translucent sample discs to form pellet and fixed followed by scanning from 4000 to 400 cm^{-1} (Kansiz et al., 2000).

4.5 Characterization of the two potent PHB producers

Characterization of the two potent PHB producers was performed by various methods like Gram staining to find out whether they are Gram positive or Gram negative, Scanning Electron Microscopy to find out their morphology, different biochemical tests to find out their sources for growth and development and Antibiotic susceptibility test to find out their sensitivity towards an antibiotic.

4.5.1 Gram Staining

Loop full cultures of the two isolates were taken on two clean, sterile glass slides and heat fixed. The heat fixed sample was then stained with primary stain crystal violet and allowed to stand for 30 sec. Excess stain was then washed off with tap water and mordant iodine was poured on the slides and allowed to stand for 30 sec. Then the slides were washed with decolourizer (ethanol) for another 30 sec followed by staining with counter stain safranin and allowed to stand for 45 sec. Then the excess stain was removed by washing the slides under tap water and air dried. Finally, the stained bacterial isolates were viewed under light microscope for morphology analysis.

4.5.2 Scanning Electron Microscope (SEM) analysis

Scanning Electron Micrographs were taken of the two isolates for morphological study as well as for size comparison of the isolates grown in both minimal and nutrient medium to confirm the production of PHB.10 ml of broth culture was taken from the test flasks. Culture was centrifuged at 8,000 g at 4˚ C for 5 minutes and then the cells were washed three times with 0.1M Phosphate Buffer Saline (KCl 0.2g/l, $KH_2PO_4$0.24g/l, NaCl 8g/l, Na_2PO_4 1.44g/l, pH- 7.0).Then the cells were fixed by adding 2% Gluteraldehyde (prepared in 0.1 M Phosphate Buffer Saline) followed by fixation of the cells by overnight incubation. Next day, cells were

washed thrice with Phosphate Buffer Saline followed by washing with 30%, 70% and 100% ethanol simultaneously. Then the fixed cells were incubated at 100% for 1hr.SEM stabs were prepared by applying adhesive tap and then applying the bacterial samples on the top (Jaysankar et al.,2008).

4.5.3 Biochemical Test

Biochemical test of performed to analyze the utilization of different carbon sources provided in the kits by the two isolates.50 µl of culture was poured into each well of the biochemical kit and the kits were incubated containing different carbon sources along with the poured culture of the two isolates for 24 h at 37° C. Similarly, remaining biochemical tests were conducted manually by using the respective culture media (Willey et al., 2008).

(1) Triple Sugar Iron test: Triple sugar iron test detects the microbe's ability to ferment sugars. 5ml of sterile Triple Sugar Iron Agar media (Peptic digest of animal tissue 10g/l, Casein enzymichydrolysate 10g/l, Yeast extract 3g/l, Beef extract 3 g/l, Lactose 10g/l, Sucrose 10g/l, Dextrose 1g/l, Sodium chloride 5g/l, Ferrous sulphate 0.20g/l, Sodium thiosulphate 0.30g/l, Phenol red 0.024g/l, Agar 12g/l, pH- 7.4±0.2) was poured in a test tube and both slant and butt were prepared. Then the bacterial culture (SE1) was swabbed inside the media using a needle and also streaked on the surface and then incubated at 37° C for 24h. The next day change was observed.

(2) Citrate utilization test: The citrate utilization test is used to differentiate enteric bacteria. The media contains sodium citrate, which serves as the carbon source and ammonium phosphate as the source of nitrogen. 5ml of simmon citrate agar media (Magnesium sulphate 0.20g/l, Ammonium dihydrogen phosphate 1g/l, Dipotassium phosphate 1g/l, Sodium citrate 2g/l, Sodium chloride 5g/l, Bromothymol blue 0.08g/l, Agar 15g/l, pH- 6.8±0.2) was poured into a test tube and swabbed the culture inside the media using a needle and incubated the culture for 24h at 37° C.

(3) Mannitol Utilization& motility test: Basically Mannitol Agar is used to differentiate pathogenic strains of Staphylococcus from non-pathogenic. Pathogenic strains of Staphylococcus ferment mannitol to form acid. In this test, 5ml of mannitol agar (Casein enzymic hydrolysate 10g/l, Potassium nitrate 1g/l, Mannitol 7.5g/l,

Phenol red 0.04g/l, Agar 3.5 g/l, pH- 7.6±0.2) was prepared and culture (SE1) was swabbed into the media followed by incubation of it for 24h at 37° C.

(4) Nitrate Reduction test: The test of nitrate reduction is done to detect a bacteria which can utilize nitrate as an electron acceptor. In the experiment, 5ml of nitrate broth (Peptic digest of animal tissue 5g/l, Meat extract 3g/l, Potassium nitrate 1g/l, Sodium chloride 30g/l, pH- 7.0±0.2) was prepared and poured into a test tube followed by inoculation of culture (SE1). The culture was then incubated at 37° C for 24h.

(5) Gelatin hydrolysis test: This test helps to detect bacteria which can synthesize a protease that can hydrolyze gelatin and can convert solid gelatin media to liquid. 5ml of Gelatin was poured into a test tube for the test and bacterial culture (SE1) was swabbed inside the media using a needle and the culture was incubated at 37° C for 24h.

(6) Urease production: It helps to detect bacteria which can produce urease enzyme which split urea to $NH_3$and CO_2. 5ml of media (Dextrose 1g/l, Peptone 1g/l, Sodium chloride 5g/l, Monopotassium phosphate 2g/l, Urea 20g/l, Phenol red 0.012g/l, pH 6.8±0.2) was prepared and poured into a test tube and then culture was swabbed inside the media by using a needle. The culture in the media was incubated at 37° C for 24h.

(7) Oxidase activity test: This test detects the presence of Cytochrome c oxidase enzyme in bacteria which can reduce O_2 and also artificial electron acceptor. For the test, oxidase activity discs were taken where culture was swabbed just at minimum volume and suddenly the change of color was observed indicating the positive result for the test.

4.5.4 Antibiotic Sensitivity Test

For antibiotic sensitivity test (Table 4) of the two isolates, 100 µl of culture was swabbed on Muller Hinton Agar Medium (HiVeg beef infusion 2g/l, HiVeg Casein acid hydrolysate 17.50g/l, Starch 1.50g/l, Agar 17 g/l, pH- 7.3±0.2). Then, 5 different types of antibiotic discs were placed on the medium and were incubated for 24 h at 37˚ C (Bauer et al., 1966).

4.6 Comparison of PHB produced in the two isolate's cell population using Flow Cytometry

The two potent PHB producers were cultured in minimal media supplemented with dextrose carbon source and after 72h of incubation comparison of PHB production in the two isolate's cell population was performed using Flow Cytometry. The cells were suspended in 1ml of phosphate buffered saline (PBS) at room temperature. FITC (Fluorescein Isothiocyanate) 490/525dissolved in DMSO (Dimethyl sulfoxide) was added to the samples and the samples with dye were incubated for 5min. The final FITC concentration was 0.038µm. After staining of the samples with FITC dye, cells were pelleted followed by resuspension in 1ml PBS and stored on ice in dark before analysis. FITC 490/525 fluorescence was measured using a band pass filter (Kacmar et al., 2005).

4.7 Molecular Analysis for amplification of genes responsible for PHB production in the two potent isolates

4.7.1 Preparation of template

For preparation of template phenol-chloroform extraction method was used where first 300 µl overnight grown bacterial culture was taken in 1.5 ml eppendorf tube. Then the culture was centrifuged at 6000rpm for 10 min followed by resuspension of the pellet in 567µl TE buffer. Then to the suspension 30µl of 10% SDS and 3µl of 20mg/ml proteinase-K were added and mixed thoroughly followed by incubation for 1h at 37˚ C. After incubation 100 µl of 5M NaCl was added and mixed thoroughly. Then, 1 volume of 24:1 Chloroform/Isolamyl alcohol was added to the suspension and mixed thoroughly and centrifuged at 6000 rpm for 5 min. The supernatant was transferred to a fresh tube. Then, 1 volume of 25:24:1 Phenol/Chloroform/Isoamyl alcohol was added to the supernatant obtained and centrifuged at 6000 rpm 5 min. Then supernatant was transferred to a fresh tube. In the next step, 0.6 volume of Isopropanol was added and mixed gently until a stringy white DNA precipitate formed. Then the suspension was centrifuged at 10000 rpm for 5 min at room temperature followed by supernatant discard and addition of 100 µl of 70% ethanol. At last, the suspension was centrifuged at 10000 rpm for 5 min and the pellet was dried until complete evaporation of ethanol followed by addition of 30 µl

of TE buffer. The purity of DNA was checked by using nanodrop and stored at -20˚ C in TE buffer till further use.

4.7.2 Descriptions of Primers used

For the amplification of *phbA, phbB* and *phbC* genes in the isolates, three primers have been used according to the report of Galehdari et al. (2009). The detailed descriptions of the primers used and their sequences have been provided in Table 1.

Table 1: Name of genes along with their sequences and Tm value.

Gene	Sequence	Tm
phbA- F	5´ATGAAAGAGGTTGTAATCGTCGCT3´	65˚ C
phbA-R	5´TCAACGCTCCACTGCGAG3´	66˚ C
phbB-F	5´ATGAGCAATCAACGAATTGCA3´	65˚ C
phbB-R	5´TCATTGCATGTTCAGACCGC3´	67˚ C
phbC-F	5´ATGGATCAAGCCCCCTCTTT3´	65˚ C
phbC-R	5´TCAGCCTTTCACGTAACGG3´	63˚ C

4.7.3 Conditions used in PCR

The PCR reaction mixture contained 5µl of each primer, 5µl of template DNA isolated from the bacterial isolates, 5 µl PCR buffer, 5µl $MgCl_2$, 1.2 µl of DNTPs and 2 µl of DNA polymerase. The cyclic conditions includes an initial denaturation of 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 2 min, annealing of 60°C for 30 sec, extension of 72°C for 2 min and a final extension of 72°C for 10 min, followed by hold at 4°C forever.

RESULTS

5.1 Isolation of various bacterial strains from marine and organic waste sources

Total 32 isolates obtained from marine (12 nos.) and organic-waste (20 nos.) sources were cultured on Nutrient agar media (Fig. 10).

Fig.10. Pure culture of the isolates from marine and organic-waste sources

5.2 Screening of the isolates for production of PHB

The isolates obtained from marine and organic wastes sources screened for PHB production using Nile blue staining method were observed under fluorescence microscope where the PHB producing colonies fluoresced bright orange (Fig. 11 and 12).

5.2.1 Marine isolates accumulating PHB

Among the marine isolates CS605 and CW603 was found to produce more PHB when observed under fluorescence microscope.

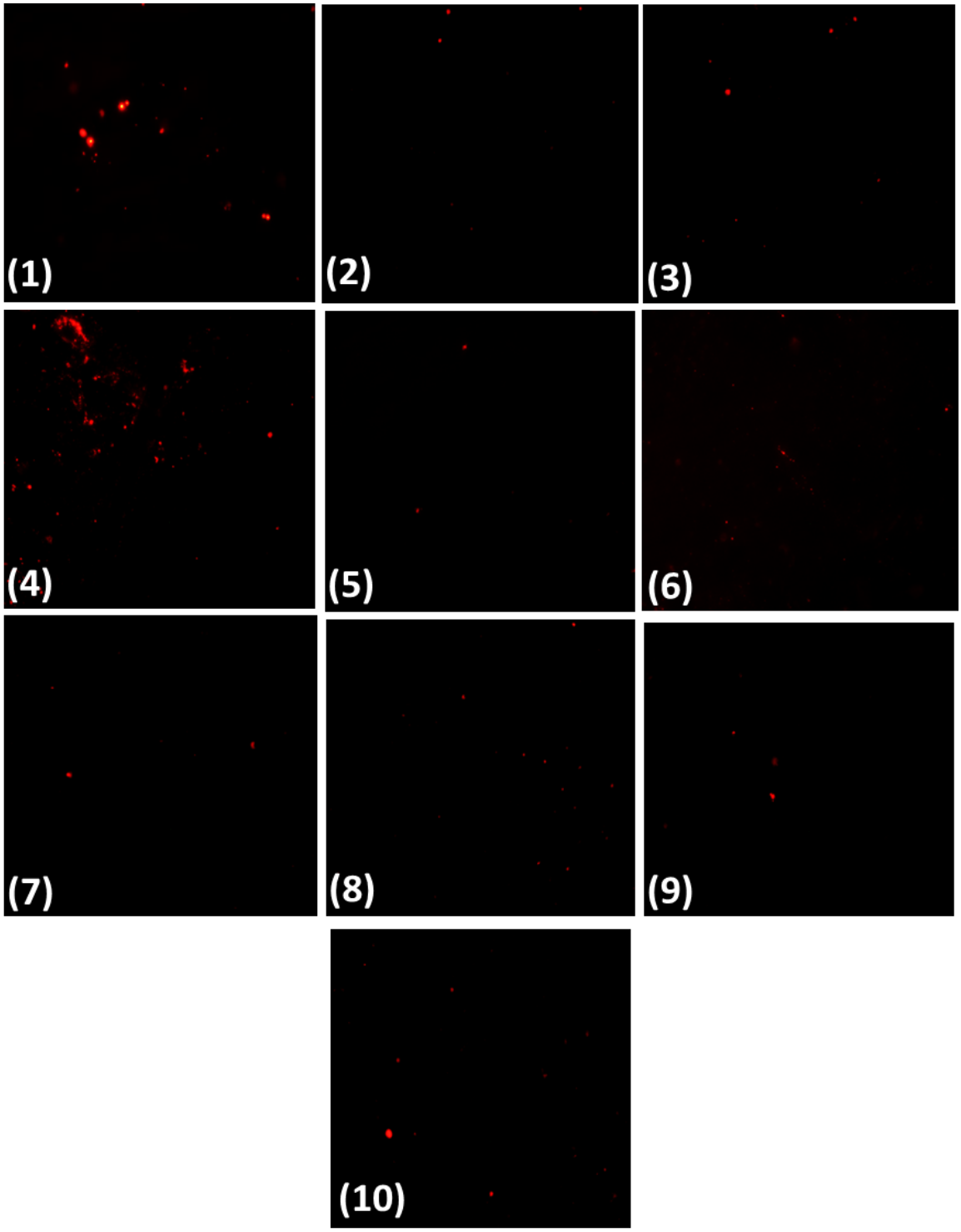
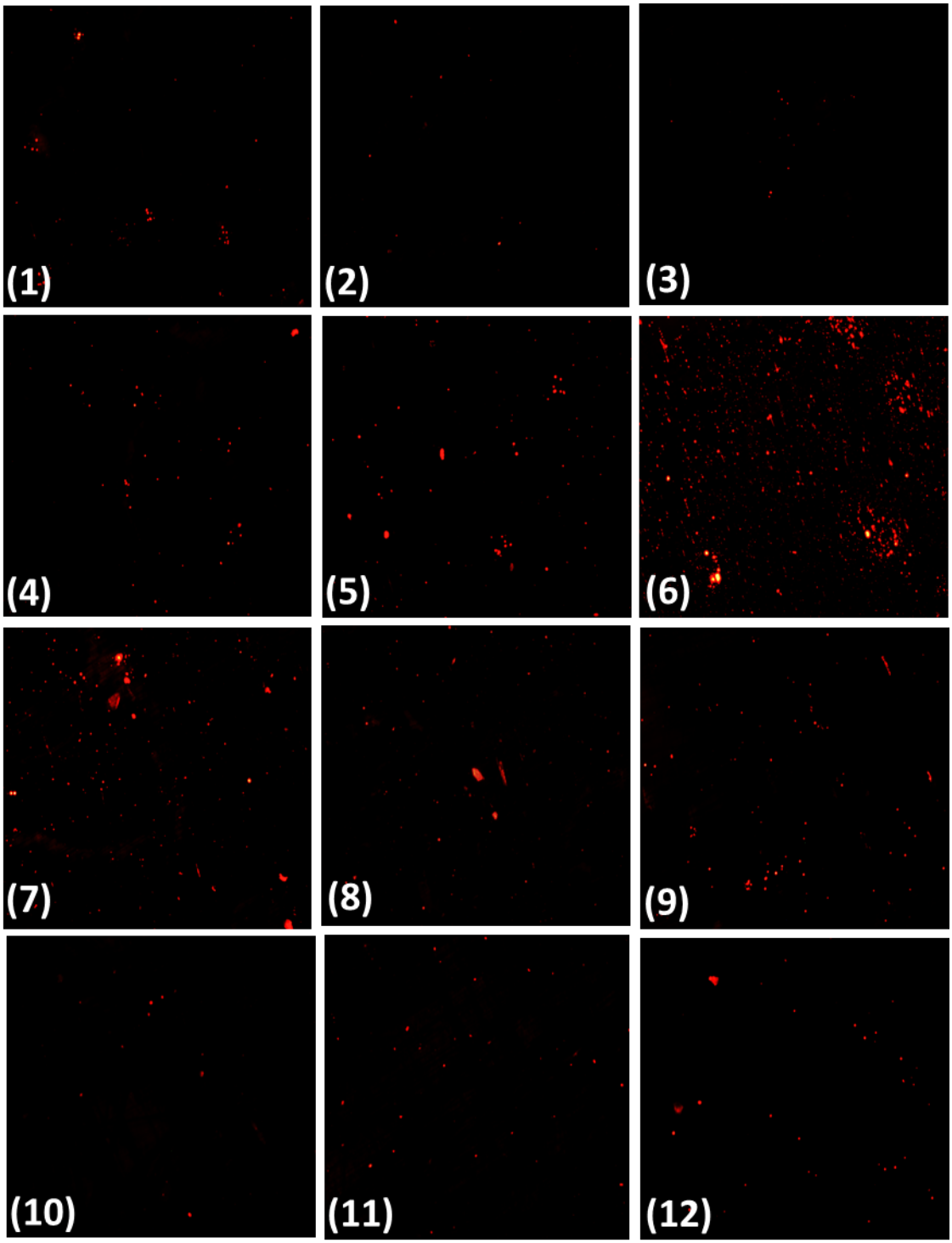


Fig.11. Different marine isolates showing positive results when screened for PHB production (1- CS605, 2- CW102, 3- CW103, 4- CW603, 5- CW605, 6- GW502, 7- PW206, 8- PW702, 9- RW202, 10- RW402)

5.2.1 Isolates of organic wastes accumulating PHB

Most of the isolates obtained from organic wastes showed the production of PHB in cells.



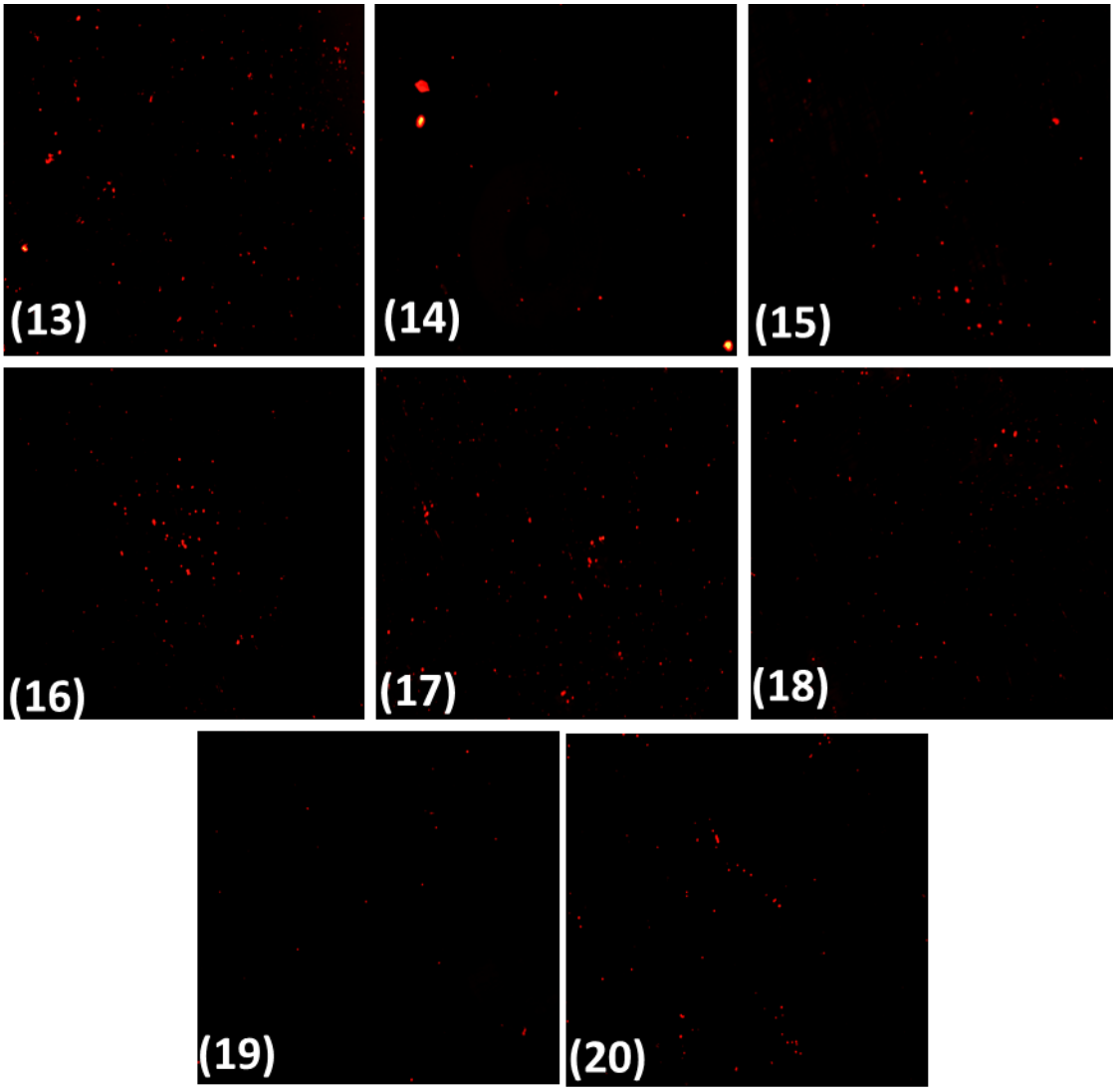


Fig.12. Isolates from organic-wastes showing positive result in screening for PHB production (1- NW1, 2- NW2, 3-NW3, 4-NW4, 5-NW5, 6-NW6, 7-NW7, 8-NW8, 9-SE1, 10-SE2, 11-SE3, 12- SE4, 13- SE5, 14- SE6, 15- SE7, 16- SE8, 17- SE9, 18- SE10, 19- SE11, 20- SE12)

5.3 Extraction of produced PHB

The extracted PHB from the two isolates CS605 and SE1using Sodium hypochlorite - Chloroform method has been shown below (Fig. 13).

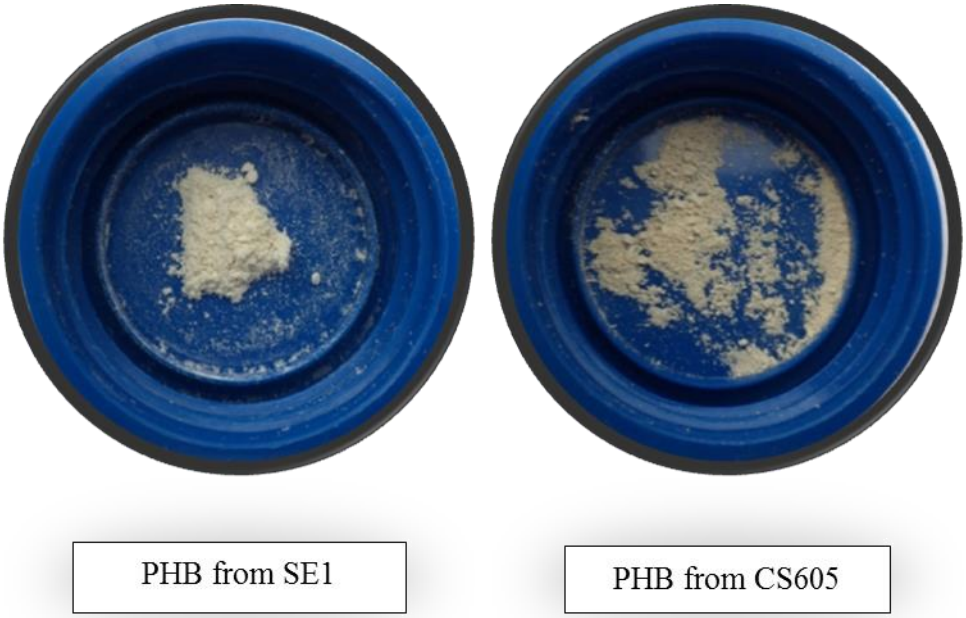


Fig.13.Extracted PHB crystals from the two isolates SE1 and CS605 by Sodium hypochlorite- Chloroform method.

5.4 FTIR analysis for characterization of extracted PHB

FTIR analysis performed for characterization of extracted PHB from the isolates resulted some peaks showing the presence of functional groups like CH_2, CH and C=O, which are also present in PHB structure (Fig. 14).

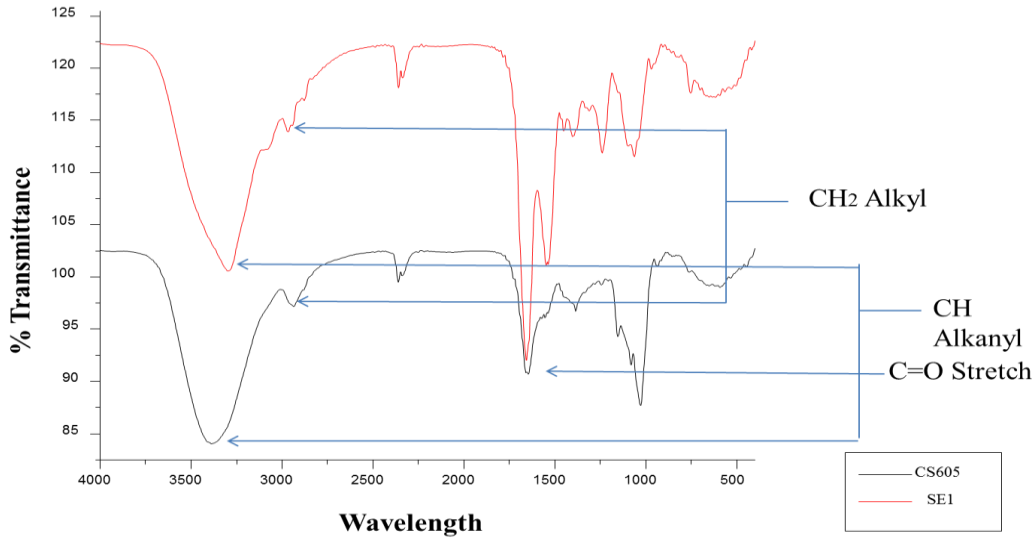


Fig.14.FTIR graph of extracted PHB from CS605 and SE1

5.5 Characterization of the potent isolates

To characterize the potent isolates SE1 and CS605 different tests like Gram staining, Scanning Electron Microscopy, Biochemical tests, Antibiotic susceptibility test were performed. The results of these tests have been presented below.

5.5.1 Gram Staining

From the Gram staining of the two potent isolates CS605 was found to be Gram positive Bacillus and SE1 was found as Gram positive Coccus (Fig. 15).

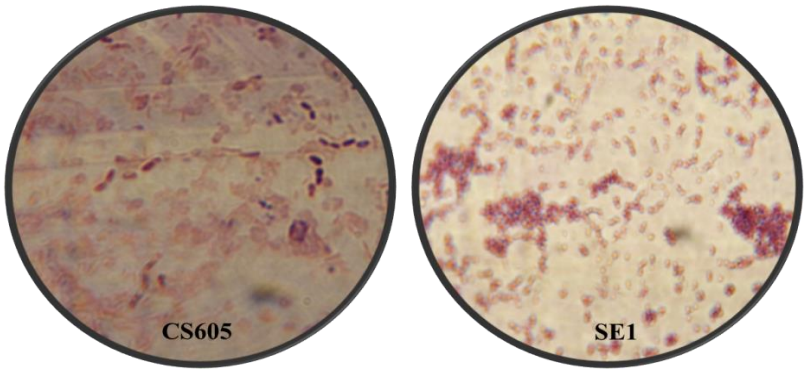


Fig.15.Gram Staining of CS605 and SE1

5.5.2 Scanning Electron Microscopy

Scanning Electron Micrographs of the two isolates grown in LB broth (Casein enzymic hydrolysate 10g/l, Yeast extract 5g/l, Sodium chloride 5g/l, pH- 7.0±0.2) media taken to find out the structural differences have been shown below in Fig. 16. From this study CS605 was found to be rod shaped and SE1 was found to be round shaped.

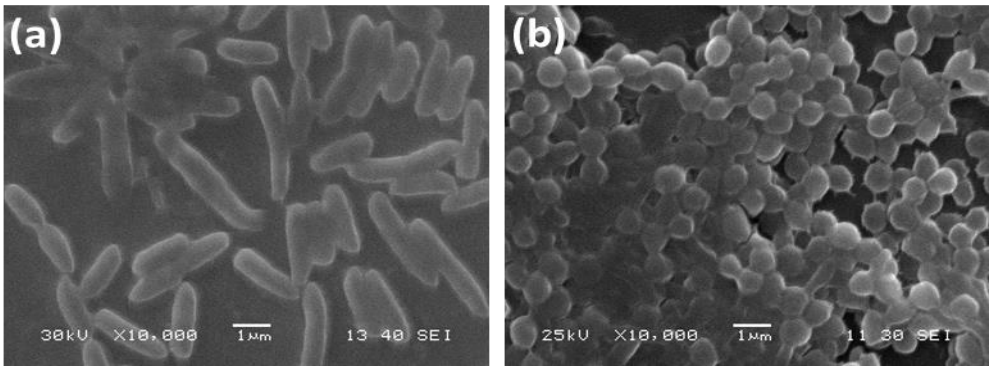


Fig.16. Scanning Electron Micrographs showing the morphology of the two isolates (a) CS605 and (b) SE1 respectively

5.5.3 Biochemical tests for identification of the two potent PHB producers

A series of biochemical tests were performed to identify the unknown potent PHB producers SE1 and CS605.

5.5.3.1 Test for utilization of different carbon sources by the potent isolate SE1

The tests for utilization of different carbon sources conducted for identification of SE1 (Fig. 17) revealed the following results listed in Table 2 (based on the changing pattern of colors).

Table 2: Different carbon source utilization tests by SE1

Tests conducted	SE1
Rhamnose	-ve
Cellubiose	+ve
Melezitose	-ve
α- Methyl-D-Mannoside	-ve
Xylitol	-ve
ONPG	-ve
Esculin	+ve
D-Arabinose	-ve
Citrate	-ve
Malonate	-ve
Sorbose	-ve
Control	-ve

Fig.17. Biochemical test for utilization of different carbon sources for identification of SE1. Tests conducted: 1-Rhamnose, 2-Cellubiose, 3-Melezitose, 4-α- Methyl-D-Mannoside, 5-Xylitol, 6-ONPG, 7-Esculin, 8-D-Arabinose, 9-Citrate, 10-Malonate, 11-Sorbose, 12-Control.

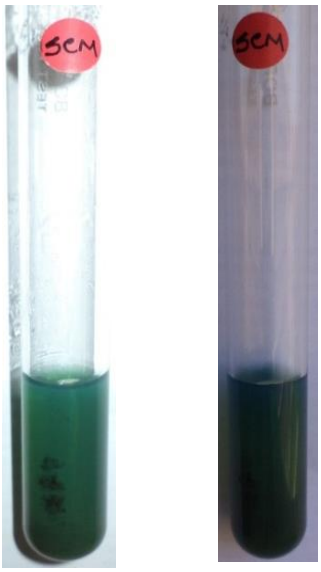
5.5.3.2 Biochemical test other than carbon source utilization for identification of SE1

(1) Triple Sugar Iron test: In this test, after 24h incubation the butt of the media containing the swabbed culture was changed to pink colored and the slant of the culture became yellow (Fig. 18).

TSI before TSI after

Fig.18.Changes in color in TSI agar medium containing bacterial culture after 24h incubation

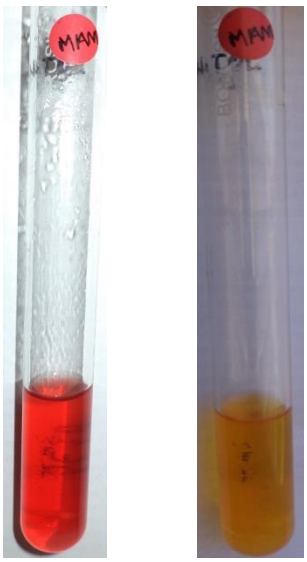
(2) Citrate utilization test: No change was observed in the citrate media containing culture (SE1) after 24h incubation period (Fig. 19).



SCM before SCM after

Fig.19.Simmon Citrate Agar medium before and after incubation with bacterial culture.

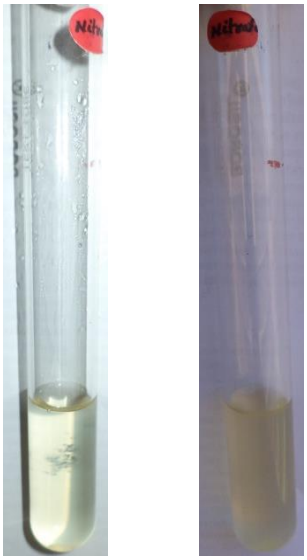
(3) Mannitol Utilization test: The color of the media after 24h incubation changed from light red to yellow (Fig. 20) giving a positive result for the test.



Mannitol before Manitol after

Fig.20. Changes in color of Mannitol Agar medium before and after incubation with bacterial culture

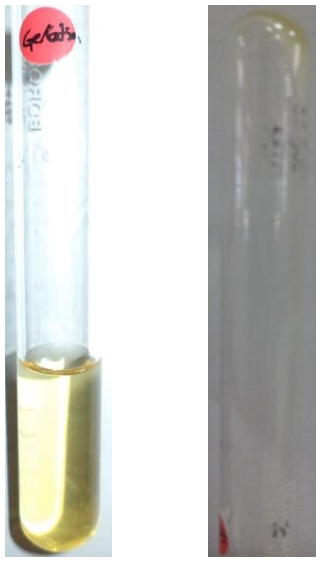
(4) Nitrate Reduction test: No specific change was observed between culture in nitrate broth before incubation and after 24h incubation (Fig. 21).



Nitrate before Nitrate after

Fig.21. Nitrate broth before and after incubation with bacterial culture

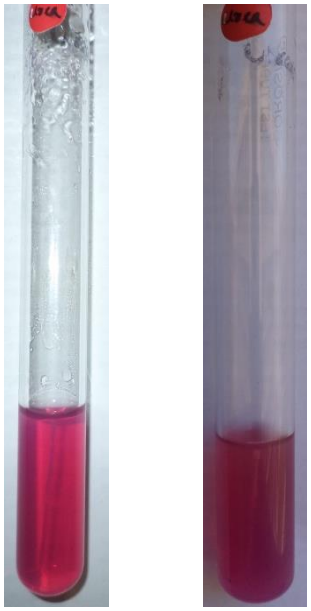
(5) Gelatin hydrolysis test: The isolate showed a positive result for gelatin hydrolysis test (Fig. 22). After incubation the culture media was found to be liquid which was semi solid before incubation.



Gelatin before Gelatin after

Fig.22.Changes in Gelatin media before and after incubation with bacterial culture

(6) Urease production: The result of urease production test was found to be negative and no specific change in culture media was observed after 24h incubation (Fig. 23).



Urea before Urea after

Fig.23. Urea Basal Medium before and after incubation with bacterial culture

(7) Oxidase activity test: This test showed SE1 negative whereas CS605 positive for oxidase activity (Fig. 24).

Fig.24. Oxidase activity test for CS605 and SE1

5.5.3.3 Biochemical tests for identification of CS605

A series of biochemical tests conducted for identification of CS605 (Fig. 25) showed the following result presented in Table 3.

Table 3: Series of biochemical tests for CS605 identification

Tests conducted	Result
Methyl red	-ve
Voges proskeur	+ve
Citrate	+ve
Esculin	+ve
Urease	+ve
ONPG	-ve
Glucose	+ve
Sucrose	+ve
Rhanmnose	+ve
Malonate	-ve
Sorbose	+ve
Control	+ve

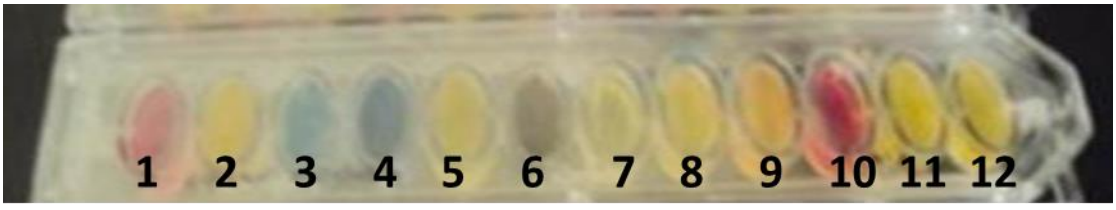


Fig.25. A series of biochemical tests for identification of CS605: Tests conducted include 1-Methyl Red, 2-Voges Proskeur, 3-Citrate, 4-ONPG, 5-Esculin,6-Urease, 7-Glucose, 8-Sucrose, 9-Ramnose, 10-Malonate, 11-Sorbose, 12- Control

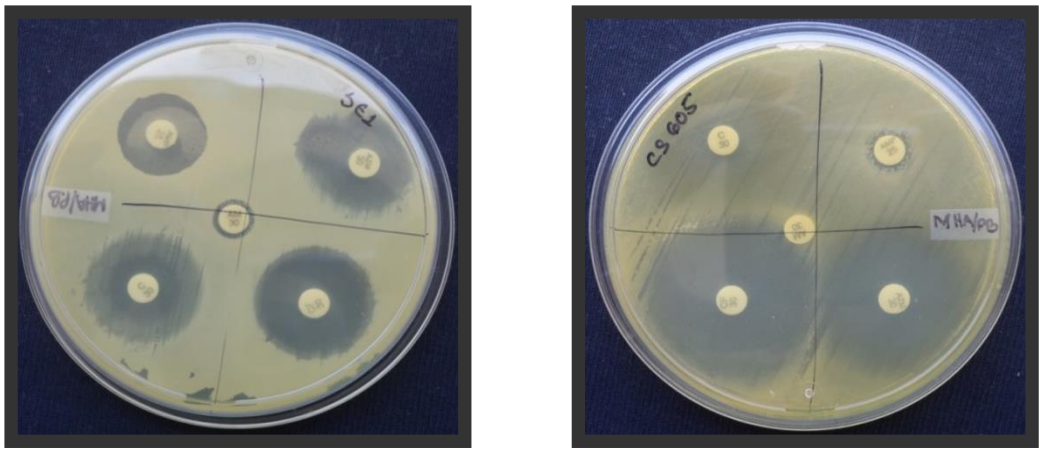
Based upon the various biochemical test results the isolates were identified to be *Bacillus* sp. and *Enterococcus camalliae* for CS-605 and SE1 respectively.

5.5.4 Antibiotic Sensitivity Test of the two isolates

Among five different types of antibiotics used in the experiment, CS605 was found to be resistant for Amoxycillin and Chloremphenicol and the zone diameters of sensitivity of the organisms to the antibiotics obtained are as follows (Table 4) (Fig. 26).

Table 4: List of antibiotics used to measure the sensitivity along with zone diameter

Antibiotics	SE1	Zone diameter (in mm.)	CS605	Zone diameter (in mm.)
Ciprofloxacin	Sensitive	0.24 m	Sensitive	0.34 mm
Amoxycillin	Sensitive	0.09 mm	Resistant	0 mm
Ampicillin	Sensitive	0.18 mm	Sensitive	0.09 mm
Chloremphenicol	Sensitive	0.23 mm	Resistant	0 mm
Azithromycin	Sensitive	0.19 mm	Sensitive	0.29 mm



SE1 (*Enterococcus camelliae*) **CS605 (*Bacillus* sp.)**

Fig.26. Antibiotic sensitivity test of SE1 and CS605

5.6 Comparison of PHB production in the two isolate's cell population using Flow Cytometry

The results of comparison of production of PHB using Flow Cytometry (Fig. 27) in the two potent isolate's cell population after 72h of incubation in minimal media supplemented with dextrose as carbon source are as follows.

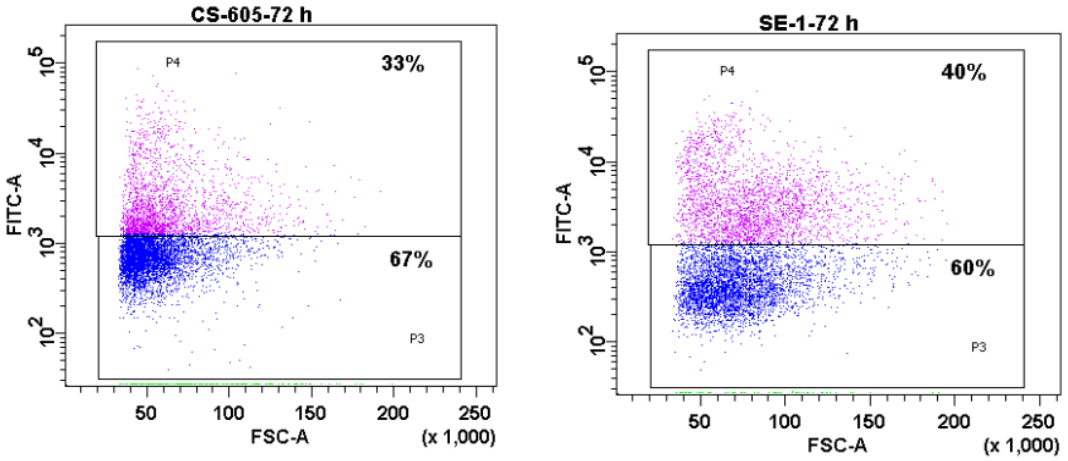


Fig.27.Comparison of PHB production in cell population of CS605 and SE1

5.7 Molecular analysis for amplification of the genes responsible for PHB production

PCR result for the amplification of *phbB* gene gave a clear banding pattern at around 1000 bp (Fig. 28) corresponding to the banding pattern observed by the previous research groups. The result signifies the presence of other functional genes like *phbA* and *phbC* as they are present in the same cascade of genes responsible for the mode of action of PHB production.

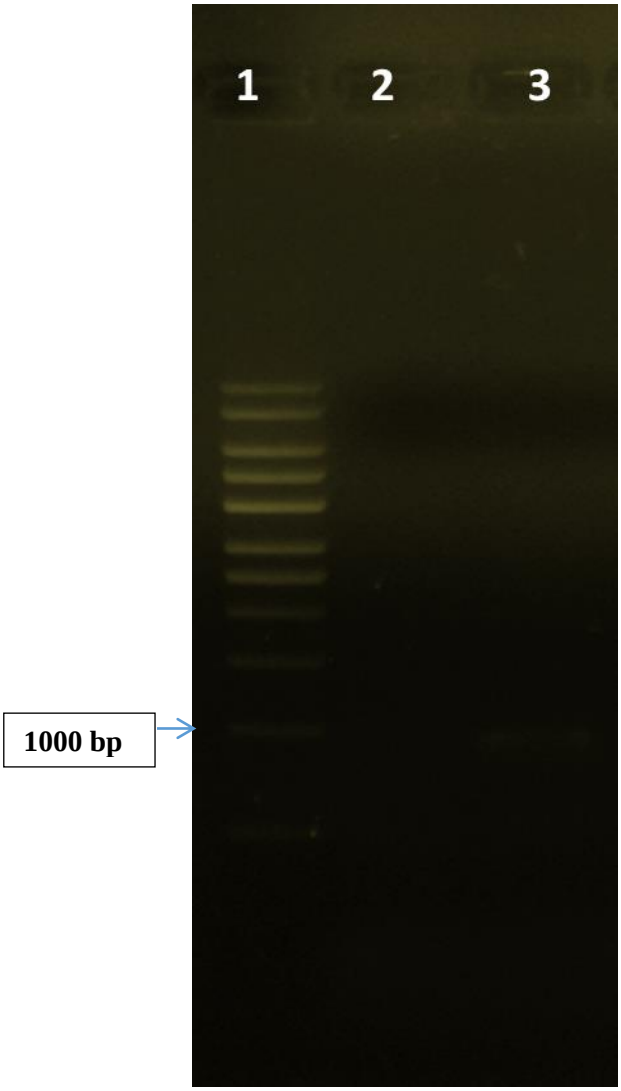


Fig.28. Amplification of *phbB* gene in the two isolates: 1- 1kb ladder, 2-CS-605, 3-SE1.

DISCUSSION

Plastic is one of the major pollutants now-a-days around the world. So, an alternative must be developed to replace this non bio-degradable pollutant, which is used by everyone in daily life for packing, carrying vegetables and for many more purposes. Though the idea of production and extraction of Bio-degradable plastic has been developed many years back but still some modification is required for large scale production in industries so that it can replace the plastic of petroleum origin. Since the production of bio-plastic is expensive many techniques have been adopted for large scale production. But, all working on this field should concentrate on the selection of proper strains of bacteria, capable of producing or accumulating PHB in large scale. Many reports are there for use of terrestrial bacteria capable of producing bioplastics (Shimamura et al., 1994; Abe et al., 1994; Saito and Doi, 1994; Fuchtenbusch et al., 2000; Numata and Doi, 2012), however, marine environments are the least explored compare to their terrestrial counterparts (Dash et al., 2013). Marine ecosystem is one of the largest ecosystems on Earth and still required to be explored. So in this work, comparison of the production of PHB (Bio- Plastic) in Marine and Soil bacteria has been done to find out which one has the potency to accumulate more PHB. Also, if in the potent isolates capable of producing PHB (Bio- Plastic) the genes responsible for its accumulation are over-expressed then also the production can be increased. *Bacillus* sp. from marine environment is found to the most potent PHB producer in the current study which is in accordance to the previous results obtained by Lopez-Cortes et al. (2008).

Precise techniques should be developed to extract PHB without any impurity so that the cost of production can be lowered. Otherwise its production would remain a dream and the gradually increasing pollution will destroy the living environment of Earth's surface. The feed stock required in fermentation is very much costly. So, at present time waste materials have been employed as biomass required for culture of microbes for PHB production.

Cloning of genes responsible for PHB production also has been performed to measure out the tendency of PHB production by some specific bacterial isolates. Different types of algae have been also studied for PHB production.

Variation of temperature, pH, and substrates also affects product formation. So the maintenance of these parameters is also very essential in large scale production of PHB in industries.

Bioplastics as mentioned earlier are biodegradable plastics. So definitely they have specific half -life period. The degradation study of PHB also should be performed to find out the life span of product made from biodegradable plastics so that in future its quality and life span can be increased.

The production of PHB has been found more in bacteria isolated from organic waste sources. The reason behind this may be the lack of resistance power of bacteria present in terrestrial and non-marine aquatic environment to adverse changes in the environment. However, the current result explored the organic waste to be the potent source of PHB producer as almost all the isolates showed the capability of producing PHB and *Enterococcus* sp. was found to be the most potent among them (Yuksekdag and Beyatli, 2008). Frequent changes occur in marine environment and thus the organisms living in marine conditions are prone to the changed conditions. So they develop adaptation to the changed environment rapidly whereas the terrestrial bacteria take some more time to adapt with the adverse changes of environment. So bacteria found in the terrestrial and non-marine environment can be believed to be more potent isolates for large scale production of bioplastic (PHB).

CONCLUSION

In the 21st century we are living in huge load of pollutions from many sources including polythene wastes, hence the search for a suitable, economical, harmless alternative is of huge demand. Bioplastics are the most suitable for this cause. Marine and organic wastes are rich in various nutrients as well as they provide many environmental stress conditions to their inhabitants which are the ultimate resources for PHB producers. In this regard, the current study revealed the presence of many PHB producers in both the environments studied which can be used for production of bioplastics in both laboratory as well as industrial scale. The characterization of PHB by various analytical techniques showed the production of pure PHB by the selected isolates which can be studied further by various blending techniques to get a more user friendly, economical goods. The most potent among the isolates were identified to be *Bacillus* sp. (CS-605) and *Enterococcus camelliae* (SE-1). *Bacillus* spp. are ubiquitous in nature and have been reported to possess the capability of overcoming the stress conditions by various mechanisms. Though *Enterococcus* spp. have been reported to be PHB producers, less study have been conducted so far in this regard. When the PHB production capability was compared between the isolates from organic wastes and marine sources, the bacteria from organic waste was found to be more capable of producing PHB which may be due to the fact that, marine microorganisms are more evolved by de novo to overcome various stress conditions. Hence, the continuous search from the various environmental conditions may provide some more suitable isolates and their genetic modification, for efficient PHB production for commercial use.

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